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The Antarctic yeast *Candida sake*: Understanding cold metabolism impact on wine

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ABSTRACT

Current winemaking trends include low-temperature fermentations and using non-*Saccharomyces* yeasts as the most promising tools to produce lower alcohol and increased aromatic complexity wines. Here we explored the oenological attributes of a *C. sake* strain, H14Cs, isolated in the sub-Antarctic region. As expected, the cold sea water yeast strain showed greater cold growth, Na^+-toxicity resistance and freeze tolerance than the *S. cerevisiae* QA23 strain, which we used as a commercial wine yeast control. *C. sake* H14Cs was found to be more sensitive to ethanol. The fermentation trials of low-sugar content must demonstrated that *C. sake* H14Cs allowed the cold-induced lag phase of growth to be eliminated and also notably reduced the ethanol (−30%) and glycerol (−50%) content in wine. Instead *C. sake* produced sorbitol as a compatible osmolyte. Finally, the inspection of the main wine volatile compounds revealed that *C. sake* produced more higher alcohols than *S. cerevisiae*. In conclusion, our work evidences that using the Antarctic *C. sake* H14Cs yeast improves low-temperature must fermentations and has the potential to provide a wine with less ethanol and also particular attributes.

1. Introduction

Grape must fermentation is a complex process that involves the sequential action of yeast from different genera and species. While early stages are dominated by the growth of non-*Saccharomyces* yeasts, *Saccharomyces cerevisiae* is imposed on subsequent days (Renault et al., 2009) given its capacity to maximise ethanol production (Piskur et al., 2006). This has been technically exploited to avoid microbial spoilage and to obtain high-alcohol wines, which have been traditionally appreciated by consumers and associated with quality products. However, consumer preferences have changed in recent years and currently there is an interest in lowering the alcohol content in wines (Varela et al., 2008, 2012). Excess ethanol negatively impacts flavour and aroma perception, increases product costs on markets where taxes are levied on alcohol concentrations and, more importantly, has negative health effects (Goldner et al., 2009; Kutyna et al., 2010). Although a range of viticultural and processing technologies have the potential to reduce alcohol in wines (Belisario-Sánchez et al., 2009; Gambuti et al., 2011), the use of non-*Saccharomyces* yeasts has been suggested as the most promising tool to produce a lower alcohol wine (Contreras et al., 2015; González et al., 2013; Quirós et al., 2014). Nevertheless, most species tested to date perform poorly or have physiological features that are not feasible with current winemaking trends.

In recent years, consumer demands have also prompted the production of wines with increasing aromatic complexity. Several approaches and strategies have been considered, including selection and improvement of grape varieties and agronomic procedures, use of enzymes, and reduction of fermentation temperatures, among others. Cold prevents loss of primary (varietal) aromas by evaporation and increases the secondary metabolism, mainly of ethyl and acetate esters (Beltrán et al., 2006; Chiva et al., 2012). However, low temperature (10–15 °C) is not the optimal growth condition for the laboratory or wine yeast strains of *S. cerevisiae* (Aguilera et al., 2007; Pizarro et al., 2008), which increases both the duration of the process and the risk of stuck or sluggish fermentations (Bisson, 1999). Although some yeast species of the *Saccharomyces* genus, like *S. kudriavzevii*, or interspecific hybrids between members of this group have been found to be better adapted to growth at low temperature than *S. cerevisiae* wine yeast strains (González et al., 2006), their use does not satisfy current demands of low-alcohol wines. Moreover, non-*Saccharomyces* yeasts appear to have a higher potential to increase the flavour fullness and complexity of wine (Steensels and Verstrepen, 2014).

More than 40 of the 1500 known yeast species have been isolated from the winemaking environment, including representatives from largely aerobic genera like *Pichia*, *Debaryomyces*, *Rhodotorula*, *Candida* and *Hansenula*, as well as yeast species with fermentative metabolism;

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e.g., *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Zygosaccharomyces bailii*. Most exhibit specific flavour-active characteristics and are known to enhance complexity, mouth feel (texture) and integration of flavours in relation to *S. cerevisiae* inoculated wines (Jolly et al., 2014). In addition, some *Metschnikowia pulcherrima* strains and two *Kluyveromyces* species have been suggested as being suitable for lowering ethanol yields by respiration (González et al., 2013; Quirós et al., 2014). Yet the usefulness of these yeasts appears to depend on the application of a high aeration regime to stimulate sugar respiration which, in turn, yields increased acetic levels (Quirós et al., 2014). Their tolerance and oenological behaviour under low-temperature conditions have still not been checked. As an alternative, non-*Saccharomyces* yeast species with a low fermentative/oxidative balance could be an obvious source of starters as either pure cultures or combined with *S. cerevisiae*. Nevertheless, the potential role of these yeasts in industrial fermentations has yet not been explored and their key oenological traits need to be tested.

Several *Candida* species have been recognised as being tolerant to winemaking conditions and to persist with *S. cerevisiae* in mixed grape juice fermentations (Ciani et al., 2010). Several authors have also reported the development of these yeast species at low temperatures in wines (Hierro et al., 2006; Maturano et al., 2015). *Candida* species have been found to positively influence the wine composition of active biomolecules, such as polysaccharides and mannoproteins (Domizio et al., 2014), which thus provides the possibility of increasing the desirable attributes of wine. Here we explored the potential oenological application of a *Candida sake* strain isolated from the Antarctic region (Carrasco et al., 2012). We hypothesised that the cold tolerance of this species, together with its low fermentative metabolism, could be advantageous features to match current wine market tendencies. This study illustrates the potential of this cryotolerant natural yeast in cold-winemaking and discusses how non-conventional yeasts might change our view about this ancient biotechnological process to lead to a functional range of wines.

2. Materials and methods

2.1. Yeast strains

The *C. sake* isolate H14Cs yeast from King George Island sea water, the major island of the Shetland South archipelago in the Sub-Antarctic region, was used throughout this work (Carrasco et al., 2012). Commercial *S. cerevisiae* wine yeast strain QA23 (Lallemand S.A., France) was used as a control.

2.2. Media, culture conditions and stress sensitivity tests

Yeast cells of *C. sake* and *S. cerevisiae* were cultured in defined media, YPD (2% yeast extract, 1% peptone and 2% glucose) or SCD [0.2% yeast nitrogen base without amino acids, 0.5% $(NH_4)_2SO_4$, 2% glucose]. Solid media contained 2% agar. Plate phenotype experiments were done by diluting the YPD overnight-grown cultures to $OD_{600} = 1.0$, and by spotting (3 μl) 10-fold serial dilutions on the same solid medium that lacked or contained 1.4 M NaCl, 1 M KCl or 9% ethanol (v/v). Yeast growth was also assayed on YP agar medium that contained 2% maltose, 2% raffinose or 3% ethanol as the sole carbon source. Unless otherwise indicated, plates were incubated for 2–7 days at the optimal growth temperature of 24 °C for the *C. sake* isolate (Carrasco et al., 2012), and at 30 °C for the *S. cerevisiae* strain. Cold growth was examined at 12–15 °C.

Yeast samples for freezing were grown in YPD liquid medium (OD_{600} ~0.5), harvested by centrifugation (3000 ×*g*, 2 min, 4 °C), washed twice with distilled water, resuspended in fresh YPD (final OD_{600} ~10.0) and 100-μl aliquots were frozen. At different time points, the cell samples of *Candida* and *Saccharomyces* were thawed for 30 min at 24 °C and 30 °C, respectively, diluted and then plated onto solid YPD. After 2 days, colonies were counted and the percent of viable cells was determined.

2.3. Fermentation trials

The cells of *C. sake* H14Cs and *S. cerevisiae* QA23 were cultivated at 24 °C and 30 °C, respectively, in YPD medium until the end of the exponential phase (OD_{600} ~10), harvested by centrifugation (3000 ×*g*, 2 min, 4 °C), washed with chilled sterile water, resuspended again in water, and used to inoculate (2×10^6 cells ml^{-1}) 60 ml of a concentrated natural must from Spanish wineries (Tempranillo 2012; 62 °Brix, pH 3.0), and kept at 4 °C. Before inoculation (2×10^6 cells ml^{-1}), the concentrated juice was diluted 5 times with distilled water. The resulting medium (pH 3.2), with a reduced sugar content of 150 g/l and a YAN of 284 mg/l, was sterilised by filtration and tryptophan was added (final concentration of 200 μM) to ensure the growth of QA23 at low temperature (Ballester-Tomás et al., 2015). Screw-cap 250-ml bottles were continuously agitated at 100 rpm and incubated at 12 °C. Fermentations were carried out at least in duplicate, and were monitored by yeast growth and sugar consumption. Fermentations were considered finished when the concentration of reducing sugars was lower than 2 g/l for QA23 or when *C. sake* was unable to further reduce sugar content. The samples taken on the last day of wine fermentation were used to determine the concentrations of polyols, ethanol and oenological attributes.

A synthetic grape must (Riou et al., 1997) was also used to test the fermentative capacity of the strains under study. The medium (pH 3.02–3.36) lacked anaerobic factors (ergosterol, oleic acid, tween-80 and uracil) and contained 200 g/l of reducing sugars (100 g/l glucose and 100 g/l fructose) and 300 mg/l of nitrogen (120 mg N/l as NH_4Cl and 180 mg N/l in the amino acid form). Tryptophan represented approximately 6.5% of the total assimilable organic nitrogen (12 mg N/l). The experiments were performed as described above. The must samples were harvested at different time points during the fermentation process to characterise growth kinetics and sugar consumption.

2.4. Analytical determinations of sugars and metabolic products

Ethanol and residual sugars (glucose and fructose) were determined enzymatically with commercial kits from Boehringer (Mannheim, Germany) and Megazyme (Wicklow, Ireland), respectively. Glycerol and sorbitol were measured by the Dionex ICS-3000 Ion Chromatography system (Thermo Scientific) coupled with pulsed amperometric detection (disposable Au on a PTFE electrode). Samples were diluted, filtered through 0.45-μm nitrocellulose membranes and injected into the system. Isocratic separation was performed at 30 °C in a CarboPac MA1 column (4 × 250 mm, DIONEX) and in a CarboPac MA1 guard column (4 × 50 mm, DIONEX). The mobile phase was 1 M of sodium hydroxide at a flow rate of 0.4 ml/min and a loop of 20 μl. Each sugar was calibrated using an external calibration curve with concentrations of each compound ranging from 0.1 to 20 g/l.

2.5. Analysis of volatiles

Extraction of higher alcohols and esters from wine samples was carried out by headspace solid-phase microextraction sampling (HS-SPME) using poly (dimethylsiloxane) (PDMS) fibres (Sigma-Aldrich, USA). At the end of fermentation, flasks were hermetically sealed for 1 h at 30 °C and 180 rpm to allow volatile compounds to accumulate in the headspace. Then the PDMS fibre was kept in the headspace for 30 min to allow the extraction of volatiles and was introduced into the injector of a Thermo Trace™ GC Ultra (Agilent Technologies, USA) for 4 min at 220 °C. For the GC analysis, an Innowax capillary-column (30 m × 0.25 mm × 0.25 μ; Agilent technologies) was coupled to a Thermo DSQ mass spectrometer. Helium was used as the carrier gas at a constant flow rate of 1.5 ml/min. The oven temperature was programmed as follows: 5 min at 60 °C, 5 °C min^{-1} to 190 °C, 20 °C min^{-1} to 250 °C, and held at this temperature for 2 min. Volatile compounds were identified by matching the mass spectra in both the Willey6 and MANILIB libraries, and by comparing the retention times with those of